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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: LIPASE VARIANTS

(57) Abstract

Lipases comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, which pocket forms part of and is surrounded by a lipid contact zone, are mutated by deletion or substitution of one or more amino acid residues in the lipid contact zone so as to change the electrostatic charge and/or hydrophobicity of the lipid contact zone or so as to change the surface conformation of the lipid contact zone of the lipases.

SUMMARY OF THE INVENTION

Further investigations have now shown that improved properties of lipases may be obtained by one or more specific mutations in the DNA sequence expressing a specific lipase in order to obtain lipase variants exhibiting such improved properties.

Consequently, in one aspect, the present invention relates to a lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, wherein the electrostatic charge and/or hydrophobicity of the lipid contact zone of the parent lipase is changed by deleting or substituting one or more negatively charged amino acid residues by neutral or positively charged amino acid residue(s), and/or by substituting one or more neutral amino acid residues by positively charged amino acid residue(s), and/or by deleting or substituting one or more hydrophilic amino acid residues by hydrophobic amino acid residue(s). For the sake of convenience, this lipase variant is termed lipase variant I in the following.

In the present context, the term "trypsin-like" is intended to indicate that the parent lipase comprises a catalytic triad at the active site corresponding to that of trypsin, i.e. the amino acids Ser, His and one of Asp, Glu, Asn or Gln. Some lipases may also comprise a surface loop structure which covers the active serine when the lipase is in inactive form (an example of such a lipase is described by Brady et al., Nature 343, 1990, pp. 767-770). When the lipase is activated, the loop structure is shifted to expose the active site residues, creating a surface with increased surface hydrophobicity which interacts with the lipid substrate at or during hydrolysis. For the present purpose, this surface is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface (or a corresponding surface of lipases which do not comprise such a loop structure). These residues may participate in lipase interaction with the

substrate at or during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface. During hydrolysis of the triglycerides, fatty acids and mono- and di-glycerides are formed in varying amounts. One reason for changing the electrostatic charge and/or hydrophobicity of the lipid contact zone by mutating the lipase in that zone is that the fatty acids formed during hydrolysis may remain in the lipid phase, thus forming a negatively charged surface. When the lipase is used for washing purposes, negatively charged detergents may form negative charges on the lipid surface. Thus, by preparing lipase variants which are less negatively charged and/or more hydrophobic, it may be possible to obtain lipases with different specificities and/or improved properties.

In another aspect, the present invention relates to a lipase variant comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, said lipase variant being further characterized by substitution, deletion, or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in the interaction with the substrate at or during hydrolysis so as to change the surface conformation of said lipid contact zone. The purpose of such a surface modification of the lipase molecule is to provide improved accessibility of the active site of the lipase to a lipid substrate. For the sake of convenience, this lipase variant is termed lipase variant II in the following.

In a still further aspect, the invention relates to a lipase variant of a type comprising (i) a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and (ii) a surface loop structure which covers the active serine

when the lipase is in inactive form and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate, the loop structure having a predominantly hydrophobic inner surface facing the binding pocket and a predominantly hydrophilic outer surface, said lipase variant being characterized by substitution, deletion or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the loop structure and/or constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in either shifting the surface loop structure or the interaction with the substrate at or during hydrolysis. This will cause the loop structure to become more open whereby the active serine becomes more accessible to the substrate. For the sake of convenience, this lipase variant is termed lipase variant III in the following.

The present invention also relates to a DNA construct comprising a DNA sequence encoding a lipase variant as indicated above, a recombinant expression vector carrying said DNA construct, a cell transformed with the DNA construct or the expression vector, as well as a method of producing a lipase variant of the invention by culturing or growing said cell under conditions conducive to the production of the lipase variant, after which the lipase variant is recovered from the culture.

The invention further relates to a detergent additive comprising a lipase variant of the invention, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme, as well as to a detergent composition comprising lipase variant of the invention.

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03. 05. 2001

(74)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
5559-WO, SLK		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00664	29/11/1999	27/11/1998
International Patent Classification (IPC) or national classification and IPC		
C12N9/20		
Applicant		
NOVOZYMES A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 13 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
31/05/2000	06.03.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
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Form PCT/IPEA/409 (cover sheet) (January 1994)

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-63 (all completely); 2-4 (all partially).

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 5-48 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☒ the claims, or said claims Nos. 5-48 are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 49-63 (completely); 2-13, 15-48 (all partially).

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

Re Item I

Basis of the report

1. The original claims 38, 40, 41, 47 and 49 were dependent on claim 27, but not on claim 21. The corresponding claims of the amended set of claims, i.e. claims 25, 27, 28, 34 and 36 are now not only dependent on claim 17 (former claim 27) but also on claim 14 (former claim 21), thereby creating added subject-matter.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

2. No opinion has been established on subject-matter which has not been searched, i.e. any subject-matter not relating to invention 1 as identified in the search report [corresponding to amended claims 1, 14 (completely) and claims 2-13, 15-48 (all partially)].

   Furthermore, no meaningful opinion could be formed on independent claim 14, because it does not clearly define the subject-matter claimed. The lipolytic enzyme of claim 14 is defined as a variant of a parent enzyme and comprising an alteration of an amino acid residue when compared to the parent enzyme. However, the parent enzyme is not clearly defined by its amino acid sequence and can be any lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position. Numerous enzymes of this kind are known from the prior art, and it is therefore impossible for the skilled reader to determine the scope of the claim. The same applies to independent claim 17. This authority considers that it is not possible to clearly define a lipolytic enzyme merely by stating that it comprises an alteration when compared to an (un-defined) "parent" lipolytic enzyme. Therefore, no meaningful opinion could be established for claims 14-48, although these claims have partially been searched.

   The independent claims of the amended set of claims now refer to an alteration of **substrate specificity** (i.e. the alteration of the **ratio of one activity to a second activity**), while the original claims referred merely to an alteration of **activity**. Dependent claims 5, 7, 8, 12, 25, 27, 28 and 34 refer to the altered substrate

254, 255, 258, 264, 265, 266 and 267 of the Rhizomucor miehei lipase or positions 203, 255, 259, 264, 265, 266 and 267 of the Humicola lanuginosa lipase are suggested in D3. The document is thus prejudicial to the novelty of claims 1-4.

Re Item VIII

Certain observations on the international application

5. The claimed invention is neither sufficiently disclosed (Article 5 PCT) nor supported by the description (Article 6 PCT), since the application fails to disclose any technical concept fit for generalisation which would enable the skilled person to alter the substrate specificity of lipolyic enzymes. The application only provides evidence that alterations in positions 266 and 259 of the Humicola lipase result in increased specificities for long-chain fatty acids, i.e. the ratio of triolein to tributyrin activity. The scope of the claims is thus not justified by the actual technical contribution to the art.

6. The application does not meet the requirements of Article 6 PCT because claims 6, 10, 14, 15 and 17 are not clear. The term "preferably" introduces ambiguity and confusion into these claims since it has in fact no limiting effect on the scope of a claim, and the feature following it is to be regarded as entirely optional.

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PCT/DK 99/00664
Applicant's ref. 5559-WO, SLK

03. 05. 2001

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AMENDED CLAIMS

In response to first written opinion

1. A method of producing a lipolytic enzyme variant comprising:

a) selecting a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position,

b) in the parent lipolytic enzyme selecting at least one amino acid residue which comprises at least one atom within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride in a three-dimensional structure of the parent lipolytic enzyme and the substrate,

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps a)-d),

f) testing substrate specificity of the variant,

g) selecting a variant having an altered substrate specificity, and

h) producing the selected variant.

2. The method of any of claim 1 wherein the parent lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Humicola* family or the *Zygomycetes* family.

3. A method of producing a lipolytic enzyme variant comprising:

a) selecting a parent lipolytic enzyme from the *Humicola* family or the *Zygomycetes* family,

b) selecting at least one amino acid residue corresponding to any of amino acids 20-25, 56-64, 81-85 and 255-269 in the *Humicola lanuginosa* lipase

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

11. The method of any of claims 8-10 wherein the alterations comprise insertion of a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

12. The method of any of claims 1-4 wherein the altered activity is a higher hydrolytic activity on a digalactosyl-diglyceride.

13. The method of claim 12 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to 21, 23, 26, 57, 62, 81, 83, 84, 85, 266, 267 or 269 in the *Humicola lanuginosa* lipase.

14. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position, which variant:

a) comprises 1-20 alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at a position which in a three-dimensional structure of the parent lipolytic enzyme and a substrate is within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride,

b) optionally comprises up to10 other amino acid alterations, and

c) has an altered substrate specificity.

15. The lipolytic enzyme of claim 14 wherein the parent lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Zygomycetes* family.

16. The lipolytic enzyme of claim 15 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109.

17. A lipolytic enzyme which:

21. The lipolytic enzyme of any of claims 17-20 which comprises a peptide extension at the N-terminal compared to the lipolytic enzyme..

22. The lipolytic enzyme of any of claims 17-21 wherein the reference lipolytic enzyme is the lipase from *Humicola lanuginosa*.

23. The lipolytic enzyme of any of claims 17-21 wherein the reference lipolytic enzyme is the lipase from *Rhizomucor miehei*.

24. The lipolytic enzyme of any of claims 17-21 wherein the reference lipolytic enzyme is the lipase from *Fusarium oxysporum*.

25. The lipolytic enzyme of any of claims 14-24 wherein the altered substrate specificity is a lower ratio of activity towards a C_4-C_8 acyl bond in a triglyceride and a C_{16}-C_{20} acyl bond in a triglyceride.

26. The lipolytic enzyme of claim 25 which comprises an amino acid alteration at a position corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266 in the *Humicola lanuginosa* lipase.

27. The lipolytic enzyme of any of claims 14-24 wherein the altered substrate specificity is a lower ratio of activity towards a C_{16}-C_{20} acyl bond in a triglyceride and a C_4-C_8 acyl bond in a triglyceride.

28. The lipolytic enzyme of any of claims 14-24 wherein the altered substrate specificity is a higher phospholipase activity.

29. The lipolytic enzyme of claim 28 which comprises an amino acid alteration at a position corresponding to R81, R84, S85, G263, L264, I265, G266, T267 or L269 in the *Humicola lanuginosa* lipase, preferably a substitution corresponding to G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; G266A, C, D, N, L, I, S, T, P, V or T267A,Q or L269N.

30. The lipolytic enzyme of claims 28 or 29 which has a phospholipase activity greater than 0.1 nmol/min in a monolayer assay at pH 5 as described herein and/or a

38. A DNA sequence encoding the lipolytic enzyme of any of claims 14-37.

39. A vector comprising the DNA sequence of claim 38.

40. A transformed host cell harboring the DNA sequence of claim 38 or the vector of claim 39.

41. A method of producing the lipolytic enzyme of any of claims 14-37 comprising
a) cultivating the cell of claim 55 so as to express and preferably secrete the lipolytic enzyme, and
b) recovering the lipolytic enzyme.

42. A process for preparing a dough or a baked product prepared from the dough which comprises adding the lipolytic enzyme of any of claims 14-37 to the dough, wherein the lipolytic enzyme preferably has phospholipase and/or digalactosyl diglyceride activity.

43. The process of claim 42 which further comprises adding to the dough an endo-amylase and/or a phospholipid.

44. The process of claim 42 or 43 wherein the endo-amylase is from *Bacillus*, and is preferably a maltogenic amylase from *B. stearothermophilus*.

45. A process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the lipolytic enzyme of any of claims 28-33 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

46. A process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid, which process comprises treating the solution or slurry with the lipolytic enzyme of any of claims 28-33 wherein the solution or slurry preferably contains a starch hydrolysate, particularly a wheat starch hydrolysate.

the second plane from the Cα atom of the active Asp residue of the parent lipolytic enzyme,

v) forming a set D consisting of atoms belonging to the union of sets B and C, and having a solvent accessibility of 15 or higher, and

vi) forming set E consisting of amino acid residues in the structure which comprise an atom belonging to set D or an atom belonging to the union of sets B and C and located less than 3.5 Å from an atom belonging to set D,

c) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than d),

e) preparing the variant resulting from steps a) -d), and

f) testing substrate specificity of the variant,

g) selecting a variant having an altered substrate specificity, and

h) producing the selected variant.

50. A method of producing a lipolytic enzyme variant comprising:

a) selecting a parent lipolytic enzyme having an active site comprising an active His residue,

b) in the amino acid sequence of the parent lipolytic enzyme selecting at least one amino acid residue at the C-terminal side of the active His residue,

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps a-d,

f) testing substrate specificity of the variant,

g) selecting a variant having an altered substrate specificity, and

h) producing the selected variant.

51. A method of producing a lipolytic enzyme variant comprising:

a) selecting a parent lipolytic enzyme,

d) preparing the lipolytic enzyme variant.

54. The method of claim 53 wherein the parent lipolytic enzyme and the amino acid to be altered are selected as defined in any of claims 1-4 or 49-52.

55. A method of preparing a lipolytic enzyme variant for use in baking, which method comprises

a) subjecting a DNA sequence encoding a lipolytic enzyme to random mutagenesis,

b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

c) screening for host cells expressing a lipolytic enzyme variant which compared to the parent lipolytic enzyme has:

i) a higher ratio selectivity for long-chain fatty acyl groups,

ii) a higher activity on digalactosyl diglyceride, and

iii) a higher phospholipase activity, and

d) preparing the lipolytic enzyme expressed by the host cells.

56. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having a lid, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue in the lid,

b) has an altered substrate specificity.

57. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having an active site comprising an active His residue, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of at least one amino acid residue at the C-terminal side of the active His residue,

b) has an altered substrate specificity.

58. A lipolytic enzyme which is a variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of at least one amino acid within 10 amino acid residues of the C-terminal,

b) has an altered substrate specificity.

b) selecting a lipolytic enzyme having hydrolytic activity towards digalactosyl diglyceride and the phospholipid, and having a ratio of activity towards the C_{16}-C_{20} acyl bond and the C_4-C_8 acyl bond which corresponds to a SLU/LU ratio of at least 3, and

c) adding the selected lipolytic enzyme to the dough.